RESEARCH PAPER

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Herbivory of maize by southern corn rootworm induces expression of the major intrinsic protein *ZmNIP1;1* and leads to the discovery of a novel aquaporin *ZmPIP2;8*

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Abbreviations: SCR, southern corn rootworm; MIP, Major intrinsic protein; NPA, Asparagine-Proline-Alanine; ar/R, aromatic/arginine; NIP, Nod-26-like intrinsic protein; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein; TM, transmembrane; RIP2, ribosome-inactivating protein; SDP, specificity determining residues

Aquaporins channel water and other neutral molecules through cell membranes. Aquaporin gene expression is subject to transcriptional control and can be modulated by factors affecting water balance such as salt, abscisic acid and drought. During infestation of maize by southern corn rootworm (SCR), an insect that chews into and significantly damages maize roots, three maize aquaporins were differentially expressed upon prolonged infestation. Using a brief infestation of maize roots *ZmNIP1;1* transcript abundance again increased under infestation while expression of a new aquaporin, *ZmPIP2;8* and *ZmTIP2;2* expression did not change. Since *ZmPIP2;8* has not been described previously, the deduced protein sequence was analyzed in silico and found to contain the hallmarks of plant aquaporins, with a predicted protein structure similar to other functionally characterized PIP2s. NIPs characterized to date have been implicated in facilitating the movement of a variety of small molecules, while TIPs and PIPs often have the capacity to facilitate trans-membrane movement of water. Functional assays (using heterologous expression in *Xenopus laevis* oocytes) of ZmTIP2;2 and ZmPIP2;8 confirmed that these aquaporins demonstrate water channel capacity.

There are at least one million species of phytophagous insects that rely on plants for their survival, so it is not surprising that plants have evolved well-orchestrated responses to herbivory. Because they are sessile organisms, plants must utilize rapid and efficient signal transduction strategies to cope with predation and ensure survival. Many microarray studies have illustrated the extensive transcriptional reprogramming a plant may experience while under insect attack.[1-7] Whole genome shifts in transcript production in response to herbivory are thought to represent reprioritization to balance basic physiological processes such as growth and reproduction with general stress related responses.[1,5,7]

Root feeders present particular challenges to the plant, as they destroy tissues involved in nutrient acquisition, storage and water balance; processes that are all essential for plant survival. Therefore, many plant responses to this underground herbivory are not only defense strategies (i.e., chemical deterrents, volatile emission etc.), but also those that affect primary metabolism, resource allocation, osmotic regulation and growth.[7] It is in osmotic regulation and growth where the ubiquitous integral membrane proteins known as Major Intrinsic Proteins (MIPs) figure. Aquaporins, the water transporting MIPs that facilitate movement of water across membranes, play key roles in osmotic regulation and growth-driving cell expansion, for review see references 8–9.

Previously, a whole genome microarray was utilized to elucidate the effects of severe southern corn rootworm *Diabrotica undecimpunctata howardi* (Barber) (SCR) infestation on the maize transcriptome.[7] In this study, differentially expressed MIPs were annotated using the Maize Genome Database (www.maizegdb.org).[10] It was found that transcript abundance corresponding to *ZmNIP1;1* was upregulated under conditions of infestation while that of *ZmTIP2;2* and a new, previously unreported *ZmPIP2* were downregulated.

MIPs are found in virtually every species of mammals, plants and microorganisms.[8,9] While the water-transporting aquaporins play a crucial role in water relations, the transport of non-water substrates such as ammonia, hydrogen peroxide and glycerol by some members of the MIP family suggests a larger role in plant

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physiology.[9] An extensive overview of 470,000 EST sequences representing 215 maize cDNA libraries identified 33 unique aquaporins.[11] Phylogenetic analysis of the maize aquaporins identified four sub-families; plasma membrane intrinsic proteins (PIPs), tonoplast membrane intrinsic proteins (TIPs), small basic intrinsic proteins and nodulin26-like intrinsic proteins (NIPs). The term NIP was coined from the first identified NIP, soybean Nod-26, which is involved in a symbiotic interaction in the peribacteroid membrane of root nodules.[9] The X intrinsic proteins, another subfamily are found in several plant taxa, but have not so far been identified in some major plant groups such as the Brassicaceae or monocots.[12-14]

Structurally, all MIPs have six trans-membrane helices separated by five loops; three extracellular (Loop 1, 3 and 5) and two intracellular (Loop 2 and 4). The helices orient longitudinally as a tetramer in the membrane to form a primary pore skeleton that substrates travel through. Inside this primary scaffold, two key areas or "constrictions" contribute to the pore architecture and substrate transport specificity. The asparagine-proline-alanine (NPA) residues in Loop 2 and 5 are hydrophilic and embed into the membrane to form one constriction. Selectivity for water is important in this area. The NPA constriction also produces a strong electrostatic barrier, effectively inhibiting the passage of protons and maintaining the important electrochemical gradient across the membrane.[8,15] The second, narrower constriction is known as the aromatic/arginine (ar/R) constriction or filter and is formed inside the molecule from only four residues; two from transmembrane (TM) helix 2 and two from Loop 5. Here substrate passage is limited by size and hydrophilic or hydrophobic character of the residues in the constriction. It is also a secondary site of proton exclusion.[9] Additional conserved amino acids also influence whether glycerol or water are transported. Froger et al.[16] identified five additional conserved amino acids P1-P5 by examining protein sequences of 40 aquaporins, which predict whether the MIP transports water or glycerol. P1 is found in loop C, P2–3 within loop E and P4–5 within TM6. The P1-P5 consensus signature of glycerol channels is (Y/F)D(K/R)P(L/M/I/A/V) while the water channels are (Q/M/E/T/A)S(S/A)(F/Y)W.

While transcriptional regulation of aquaporins in plants by abiotic stress has been well established,[9] less is known about transcriptional regulation of aquaporins by biotic stresses, particularly insect herbivory. Here, we assess transcriptional response of three aquaporins by SCR herbivory, one of which has not been previously described. In silico analyses were used to identify conserved aquaporin motifs in the sequence of the new PIP2, designated ZmPIP2;8, and *Xenopus* oocyte swelling assays used to assess the water channel function of ZmPIP2;8 and ZmTIP2;2.

SCR Infestation Response of Maize Aquaporin Transcripts

Previous microarray results demonstrated that infestation for four weeks induced *ZmNIP1;1* and repressed *ZmTIP2;2* and *ZmPIP2*.[7] Infestations were performed on 7-d-old maize plants with six second to third instar larvae for 2 to 4 d. Using qRT-PCR, the three aquaporin genes were assayed (**Fig. 1**) as was a ribosome inactivating protein (*ZmRIP2*). Although ZmRIP2 is not a MIP it has been previously characterized as an insect defense gene in maize and therefore used to confirm a defense response to this brief infestation.[17] *ZmRIP2* and *ZmNIP1;1* transcripts were induced by infestation. However the *ZmTIP2;2* and *ZmPIP2;8* were not differentially expressed by this brief infestation.

ZmPIP2;8 Amino Acid Sequence Structure

The ZmPIP2 sequence was predicted to have six α helices and 5 loops with loop B and D inside and loops A, C and E outside the cell. Both trans-membrane domain-predicting programs generate similar proteins, confirming the structure of a typical PIP. However each program places the helices at slightly different locations (**Table 1**). An amino acid sequence alignment of all 8 ZmPIP2s is shown (**Fig. 2**) and highlights the conserved NPA domains of loop B and E, which are thought to form the pore[11] along with an invariant H residue in loop D, the Ar/R filter is also shown. The TM domains are also marked (**Fig. 2**), using information from Hove and Bhave[18] and the results from the TM domain programs as a guide. The similarity of this novel PIP to the other ZmPIP2s varies from 83–87%. Comparison to ZmPIP1 proteins show at best 80% similarity to ZmPIP1;3/1;4. Since seven ZmPIP2 proteins have been described in maize,[11] this 283 amino acid protein was named ZmPIP2;8. A phylogenetic tree comparing ZmPIP2;8 to other PIP2 proteins from maize was constructed (**Fig. 3**). While ZmPIP2;8 is most closely associated with ZmPIP2;2 it is clearly a unique ZmPIP2.

Functional Assay of ZmTIP2;2 and ZmPIP2;8

The *ZmTIP2;2* and *ZmPIP2;8* clones were assayed for their protein's capacity to channel water using cRNA injection of *Xenopus* oocytes as described in the "Materials and Methods." Neither of these proteins has been tested previously for this function. The results were compared with the strong water permeability of oocytes injected with cRNA of *L. bicolor* aquaporin JQ585595 as the positive control (**Fig. 4**). The P_f values for oocytes microinjected with cRNAs of *ZmPIP2;8* and *ZmTIP2;2* were respectively, 11- and 6.1-fold higher than those of oocytes injected with water, showing ZmPIP2;8 and ZmTIP2;2 were functional aquaporins with significant water transport capacity in this heterogonous expression system (**Fig. 4**). The P_f value of the protein corresponding to ZmPIP2;8 was significantly higher than that corresponding to ZmTIP2;2.

In experiments for the microarray, roots were exposed to SCR larvae for a longer period of time. Wilting and browning of those plant shoots may indicate a downregulation of water transporters such as ZmPIP2;8 and ZmTIP2;2, which can significantly change hydraulic conductivity.[9]

Brief infestation does not have a similar effect on these transporters nor does it result in any obvious shoot stress. Further studies are required to correlate the role of infestation on the water transporters ZmTIP2;2 and ZmPIP2;8. The induction of *ZmNIP1;1* under both a brief and prolonged infestation may suggest that it plays a different role during herbivory other than

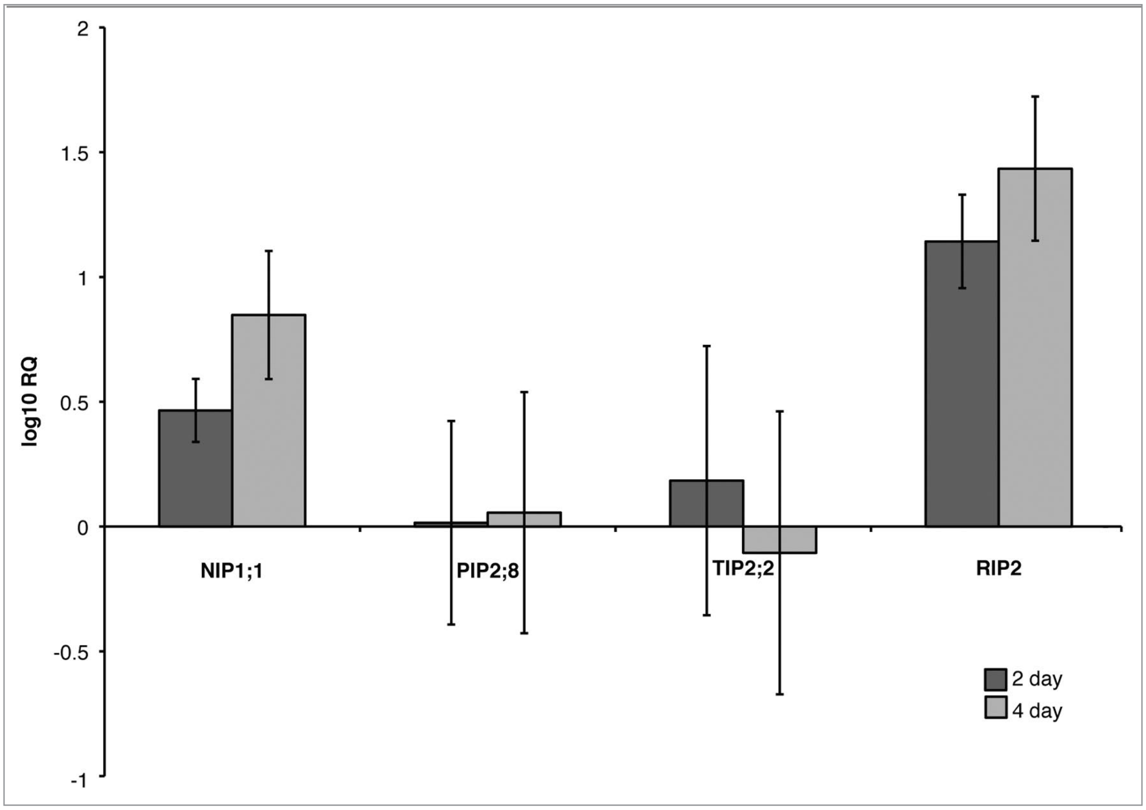


Figure 1. SCR infestation of maize roots significantly induced *ZmNIP1;1* and *ZmRIP2* (AAF60304) but did not affect *ZmPIP2;8* and *ZmTIP2;2*. Plants were grown, harvested and RNA was isolated from maize roots. cDNA was synthesized and qRT-PCR performed as described in Lawrence et al.[7] Relative Quantity (RQ) was calculated where control uninfested RNA at time 0 was designated as the calibrator and scaled to a value of 1. The data are expressed as $\log_{10}$; the $\log_{10}$ transformed value of the control is zero. Error bars represent standard deviation. Three biological replicates are averaged for each time point. Four plants were pooled for each biological replicate.

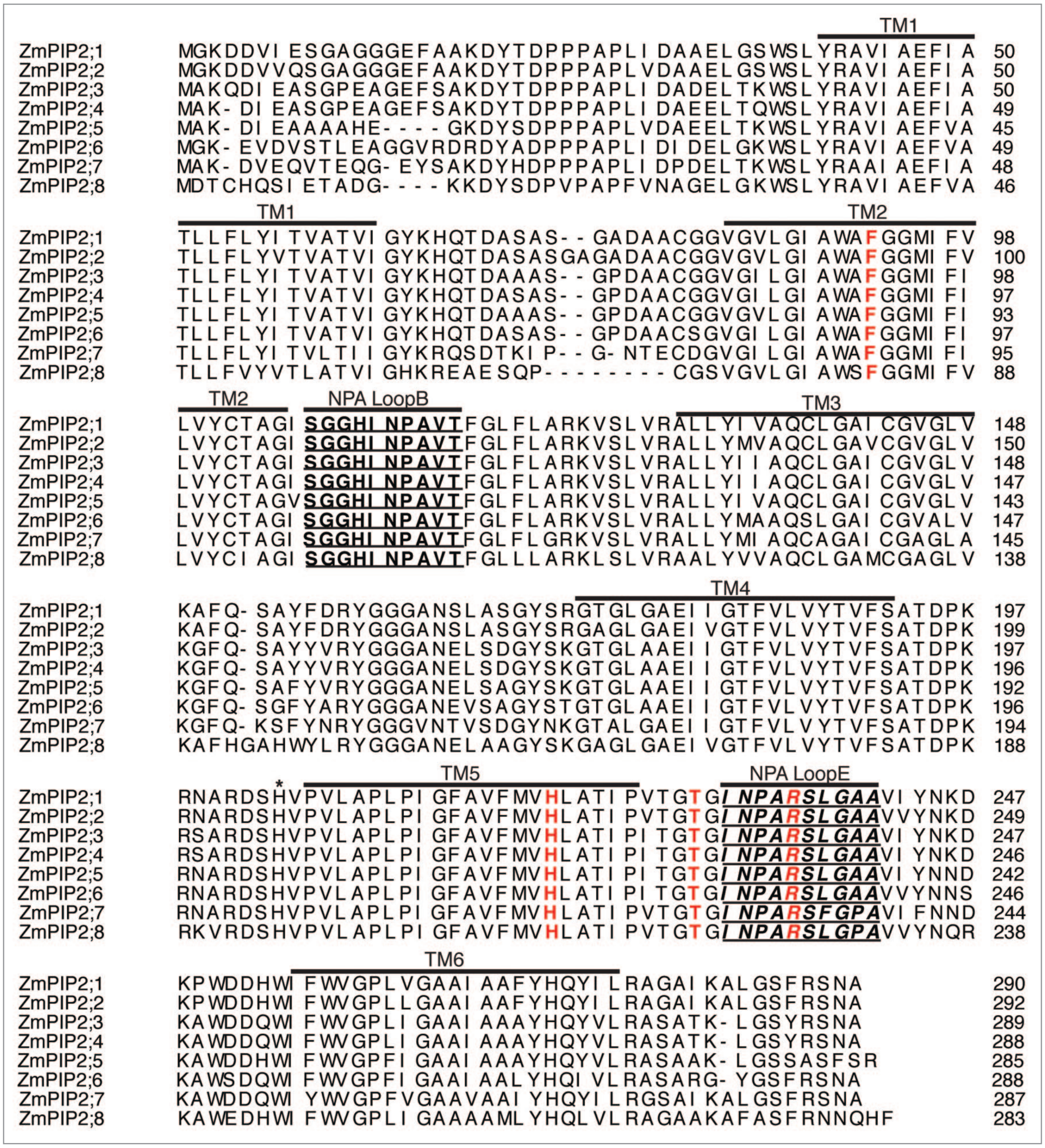
responding to changes in osmotic balance. Perhaps this MIP is transporting an alternative substrate involved in the plant's defense response.

The conserved amino acids of the MIPs affected by SCR infestation were examined. Considering that the ZmTIP2;2 and the ZmPIP2;8 transport water, do these proteins contain the conserved amino acids from the Ar/R filter or P1-P5 residues with the properties of a water channeling aquaporin? A comparison of conserved amino acids of the MIPs to AQP1 the prototypical water channel protein and GlpF the prototypical glycerol transporter (**Table 2**) shows that in the Ar/R filter and the P1-P5 residues of ZmPIP2;8 and ZmTIP2;2 share six and seven of the nine residues respectively with the water channel protein AQP1. Although the Ar/R filter contains two nonpolar neutral residues at the second and third position similar to GlpF, all P1-P5 residues of ZmTIP2;2 are common to water channel proteins such as AQP1.[16] The ZmPIP2;8 differs from AQP1 only at the third position of the Ar/R filter, which is also a T for all the other ZmPIP2 proteins and the P1 residue has an H rather than T for AQP1 or an invariant Q for the other ZmPIP2s. This last difference is a change from what is a polar neutral amino acid for all the ZmPIP2s to a basic residue for ZmPIP2;8. These differences from AQP1 however still allowed functional water transport in *Xenopus* oocytes (**Fig. 4**).

Table 1. Prediction of secondary structure of ZmPIP2;8 according to TMHMM version 2.0 and HMMTOP version 2.0

	TMHMM	HMMTOP	
intracytoplasmic	1–35	1–39	N-terminal
TM1	36–58	40–59	Helix 1
extracytoplasmic	59–72	60–85	Loop A
TM2	73–95	86–107	Helix 2
intracytoplasmic	96–115	108–119	Loop B
TM3	116–138	120–138	Helix 3
extracytoplasmic	139–163	139–164	Loop C
TM4	164–183	165–185	Helix 4
intracytoplasmic	184–195	186–197	Loop D
TM5	196–218	198–218	Helix 5
extracytoplasmic	219–243	219–244	Loop E
TM6	244–266	245–266	Helix 6
intracytoplasmic	267–283	267–283	C-terminal

ZmPIP2;8 is 283 amino acids and the numbers represent the position of the protein sequence within each domain as predicted by the different programs. Both programs predict six trans-membrane helices and that the N-terminal sequence and the C-terminal sequence reside inside (intracytoplasmic) the cytoplasm along with loop B and D, while loop A, C and E are outside (extracytoplasmic) the membrane.



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ZmPIP2;1   MGKDDVIESGAGGGEFAAKDYTDPPPAPLIDAAELGSWSLYRAVIAEFIA   50
ZmPIP2;2   MGKDDVVQSGAGGGEFAAKDYTDPPPAPLVDAAELGSWSLYRAVIAEFIA   50
ZmPIP2;3   MAKQDIEASGPEAGEFSAKDYTDPPPAPLIDADELTKWSLYRAVIAEFIA   50
ZmPIP2;4   MAK-DIEASGPEAGEFSAKDYTDPPPAPLIDAEELTQWSLYRAVIAEFIA   49
ZmPIP2;5   MAK-DIEAAAAHE----GKDYSDPPPAPLVDAEELTKWSLYRAVIAEFVA   45
ZmPIP2;6   MGK-EVDVSTLEAGGVRDRDYADPPPAPLIDIDELGKWSLYRAVIAEFVA   49
ZmPIP2;7   MAK-DVEQVTEQG-EYSAKDYHDPPPAPLIDPDELTKWSLYRAAIAEFIA   48
ZmPIP2;8   MDTCHQSIETADG----KKDYSDPVPAPFVNAGELGKWSLYRAVIAEFVA   46

ZmPIP2;1   TLLFLYITVATVIGYKHQTDASAS--GADAACGGVGVLGIAWAFGGMIFV   98
ZmPIP2;2   TLLFLYVTVATVIGYKHQTDASASGAGADAACGGVGVLGIAWAFGGMIFV  100
ZmPIP2;3   TLLFLYITVATVIGYKHQTDAAAS--GPDAACGGVGILGIAWAFGGMIFI   98
ZmPIP2;4   TLLFLYITVATVIGYKHQTDASAS--GPDAACGGVGILGIAWAFGGMIFI   97
ZmPIP2;5   TLLFLYITVATVIGYKHQTDAAAS--GPDAACGGVGVLGIAWAFGGMIFI   93
ZmPIP2;6   TLLFLYITVATVIGYKHQTDASAS--GPDAACSGVGILGIAWAFGGMIFI   97
ZmPIP2;7   TLLFLYITVLTIIGYKRQSDTKIP--G-NTECDGVGILGIAWAFGGMIFI   95
ZmPIP2;8   TLLFVYVTLATVIGHKREAESQP--------CGSVGVLGIAWSFGGMIFV   88

ZmPIP2;1   LVYCTAGISGGHINPAVTFGLFLARKVSLVRALLYIVAQCLGAICGVGLV  148
ZmPIP2;2   LVYCTAGISGGHINPAVTFGLFLARKVSLVRALLYMVAQCLGAVCGVGLV  150
ZmPIP2;3   LVYCTAGISGGHINPAVTFGLFLARKVSLVRALLYIIAQCLGAICGVGLV  148
ZmPIP2;4   LVYCTAGISGGHINPAVTFGLFLARKVSLVRALLYIIAQCLGAICGVGLV  147
ZmPIP2;5   LVYCTAGVSGGHINPAVTFGLFLARKVSLVRALLYIVAQCLGAICGVGLV  143
ZmPIP2;6   LVYCTAGISGGHINPAVTFGLFLARKVSLVRALLYMAAQSLGAICGVALV  147
ZmPIP2;7   LVYCTAGISGGHINPAVTFGLFLGRKVSLVRALLYMIAQCAGAICGAGLA  145
ZmPIP2;8   LVYCIAGISGGHINPAVTFGLLLARKLSLVRAALYVVAQCLGAMCGAGLV  138

ZmPIP2;1   KAFQ-SAYFDRYGGGANSLASGYSRGTGLGAEIIGTFVLVYTVFSATDPK  197
ZmPIP2;2   KAFQ-SAYFDRYGGGANSLASGYSRGAGLGAEIVGTFVLVYTVFSATDPK  199
ZmPIP2;3   KGFQ-SAYYVRYGGGANELSDGYSKGTGLAAEIIGTFVLVYTVFSATDPK  197
ZmPIP2;4   KGFQ-SAYYVRYGGGANELSDGYSKGTGLAAEIIGTFVLVYTVFSATDPK  196
ZmPIP2;5   KGFQ-SAFYVRYGGGANELSAGYSKGTGLAAEIIGTFVLVYTVFSATDPK  192
ZmPIP2;6   KGFQ-SGFYARYGGGANEVSAGYSTGTGLAAEIIGTFVLVYTVFSATDPK  196
ZmPIP2;7   KGFQ-KSFYNRYGGGVNTVSDGYNKGTALGAEIIGTFVLVYTVFSATDPK  194
ZmPIP2;8   KAFHGAHWYLRYGGGANELAAGYSKGAGLGAEIVGTFVLVYTVFSATDPK  188

ZmPIP2;1   RNARDSHVPVLAPLPIGFAVFMVHLATIPVTGTGINPARSLGAAVIYNKD  247
ZmPIP2;2   RNARDSHVPVLAPLPIGFAVFMVHLATIPVTGTGINPARSLGAAVVYNKD  249
ZmPIP2;3   RSARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSLGAAVIYNKD  247
ZmPIP2;4   RSARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSLGAAVIYNKD  246
ZmPIP2;5   RNARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSLGAAVIYNND  242
ZmPIP2;6   RNARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSLGAAVVYNNS  246
ZmPIP2;7   RNARDSHVPVLAPLPIGFAVFMVHLATIPVTGTGINPARSFGPAVIFNND  244
ZmPIP2;8   RKVRDSHVPVLAPLPIGFAVFMVHLATIPVTGTGINPARSLGPAVVYNQR  238

ZmPIP2;1   KPWDDHWIFWVGPLVGAAIAAFYHQYILRAGAIKALGSFRSNA         290
ZmPIP2;2   KPWDDHWIFWVGPLLGAAIAAFYHQYILRAGAIKALGSFRSNA         292
ZmPIP2;3   KAWDDQWIFWVGPLIGAAIAAAYHQYVLRASATK-LGSYRSNA         289
ZmPIP2;4   KAWDDQWIFWVGPLIGAAIAAAYHQYVLRASATK-LGSYRSNA         288
ZmPIP2;5   KAWDDHWIFWVGPFIGAAIAAAYHQYVLRASAAK-LGSSASFSR        285
ZmPIP2;6   KAWSDQWIFWVGPFIGAAIAALYHQIVLRASARG-YGSFRSNA         288
ZmPIP2;7   KAWDDQWIYWVGPFVGAAVAAIYHQYILRGSAIKALGSFRSNA         287
ZmPIP2;8   KAWEDHWIFWVGPLIGAAAAMLYHQLVLRAGAAKAFASFRNNQHF       283
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Figure 2. Alignment of ZmPIP2;8 and other ZmPIP2 sequences showing the conserved NPA domains of loop B in bold, loop E in bold italics and both underlined.[11] The transmembrane domains are labeled TM1-6. The conserved H of loop D is starred. The conserved Ar/R filter is in red. ZmPIP2;1(AAK26758.1), ZmPIP2;2 (AAK26759.1), ZmPIP2;3 (AAK26760.1), ZmPIP2;4 (AAK26761.1), ZmPIP2;5 (AAD28761.1), ZmPIP2;6 (AAK26762.1), ZmPIP2;7 (AAK26763.1).

A closer look at the conserved amino acids involved in substrate-specific transport by NIPs may suggest what this MIP may be transporting. NIPs can be subdivided into three groups based on protein sequence[19] and ZmNIP1;1 is in group I along with the first described NIP, GmNod26 from soybean. First the four residues of the Ar/R filter are conserved between GmNod26 and ZmNIP1;1 and second the Ar/R filter of NIP1s are more similar to GlpF (**Table 2**). In fact all the NIP1s have the same Ar/R motifs except AtNIP3;1, which has an I at the second position, which is also an aliphatic amino acid (**Table 2**). The ZmNIP1;1 protein sequence has a hybrid P1-P5 residues partly consisting of the water channeling aquaporin and the glycerol channeling aquaporin (**Table 2**). All the NIP1s have glycerol like residues at P1 and P5, but the water channeling type at P2-P4. Froger et al.[16] mentions this hybrid type in their analysis of NLM1, later renamed AtNIP1;1. All of the NIP1s (**Table 2**) have invariant P1 (F), P3 (A) and P4 (Y). At P2 all were S except OsNIP1;3, which was a T, still a neutral amino acid. P5 had the greatest amount of variability, V, I or L all aliphatic residues. AtNIP1;1, AtNIP1;2 and GmNOD26 have been shown to transport glycerol (as reported in).[18] However GmNOD26 also transports ammonia and formamide. AtNIP1;1 also transports arsenite and AtNIP1;2 also transports H_2O_2.[20]

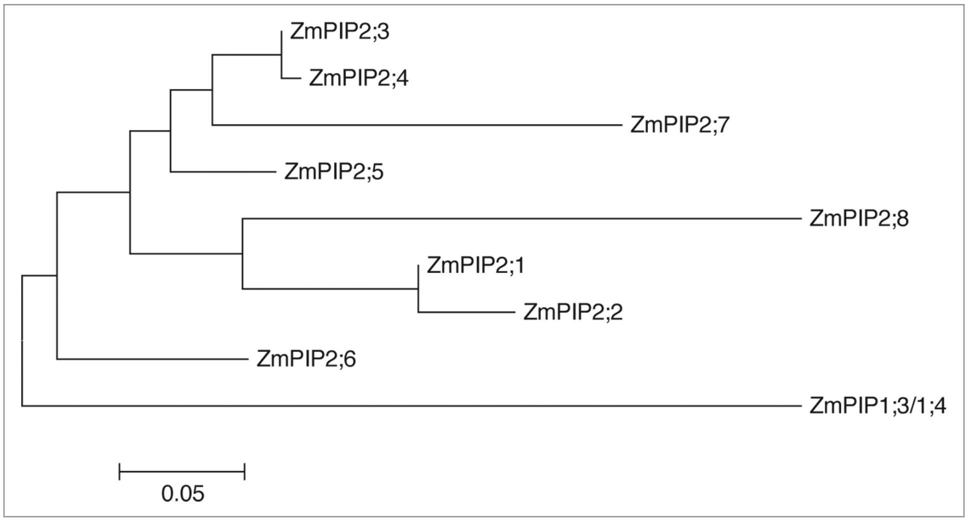

Figure 3. ZmPIP2;8 clusters with other ZmPIP2s in a phylogenetic tree comparing ZmPIP2;1(AAK26758.1), ZmPIP2;2 (AAK26759.1), ZmPIP2;3 (AAK26760.1), ZmPIP2;4 (AAK26761.1), ZmPIP2;5 (AAD28761.1), ZmPIP2;6 (AAK26762.1), ZmPIP2;7 (AAK26763.1), ZmPIP1;3/1;4 (Q9AQU5.1). The maximum likelihood method was used as described in the materials and methods.

Is there a physiological role for an increase of *ZmNIP1;1* transcript and the transport of any of these non-water compounds during herbivory? Of the neutral molecules listed above H_2O_2 has been linked to herbivory. It is induced systemically by wounding and acts as a second messenger for the systemic induction of defense genes in tomato.[21] It has been shown to play a role in early response to wounding and other stresses in *Arabidopsis*.[22] In fact several studies show that many genes induced by infestation with either phloem feeding or chewing insects are also induced by oxidative stress.[23] Finally, the maize inbred line MP708, which is resistant to several lepidopteran larvae have higher levels of JA, as well as higher levels of several JA responsive defense genes, higher levels of transcript for the enzyme, NADPH oxidase that synthesizes H_2O_2 and also higher levels of H_2O_2 upon herbivory in comparison to a susceptible inbred line.[24] Perhaps upon SCR infestation of maize *ZmNIP1;1* is induced to transport an increased amount of H_2O_2 within the plant. Further work will be needed to confirm this prediction.

Hove and Bhave[18] identified several signatures for H_2O_2 transporters within the Ar/R filter, P1-P5 residues and additional specificity determining residues (SDP). The consensus signatures for H_2O_2 transporters are as follows: Ar/R filter (H/F/W), (I/H/V), (A/T/G), (V/R); P1-P5 residues (T/Q/F), (A/S), A, (Y/F), (W/I); SDPs (S/A), (A/G), (L/V), (A/V/F/L), (I/V), (H/I/Q/L), (A/V), P.[18] The Ar/R filter of the H_2O_2 transporters could contain any of the amino acid residues found in the NIP1s, ZmPIP2;8 and ZmTIP2;2. However the P1-P5 residues found in the H_2O_2 transporters differs in one position in 6 of 10 NIP1s and one in the ZmPIP2;8. The P5 consensus residue in H_2O_2 transporters is either W/I, which is found in only five of ten NIP1s. Comparing the NIP1s, ZmPIP2;8 and ZmTIP2;2 for the H_2O_2 transporter SDPs shows the following. The consensus residues in the first, second, third, fifth and eighth positions are present in all the NIP1 proteins examined as well as in ZmPIP2;8 and ZmTIP2;2 (**Table 2**). The remaining three positions vary from the consensus signature for a H_2O_2 transporting MIP at only one residue of the SDPs in seven of the ten NIP1s examined (**Table 2**). ZmPIP2;8, AtNIP1;1, AtNIP1;2 and AtNIP3;1 contain all H_2O_2 transporters consensus SDPs. While ZmTIP2;2 differs with the consensus SDPs at the fourth and sixth position.

Since AtNIP1;2 has been shown to transport H_2O_2[20] are there other NIP1s with the conserved amino acids that predict they also transport H_2O_2? Comparison of the NIP1s and ZmTIP2;2 and ZmPIP2;8 (**Table 2**) shows that AtNIP3;1 does not vary in any of these positions from any of the known H_2O_2 MIPs. AtNIP1;1, OsNIP1;1, OsNIP1;4 and ZmPIP2;8 varies in only one of these conserved amino acids. ZmNIP1;1, OsNIP1;2, OsNIP1;3, GmNOD26, AtNIP2;1 and ZmTIP2;2 vary in only two of the amino acids. Perhaps this natural variation in MIPs could be used to test the in silico results identifying important conserved amino acids.

Finally, this study also describes a new aquaporin that had been overlooked in previous maize aquaporin research, but is not unique to the maize variety used in this study, since it is present in the sequenced and annotated maize genome from the well studied maize variety B73. It contains the typical structural aspects of an aquaporin with six trans-membrane domains, the conserved NPA motifs and Ar/R filter that have been shown to be critical for the water channel in other well characterized aquaporins (**Fig. 2**, **Table 1**). The only difference in the conserved E loop between ZmPIP2s is an A vs. a P respectively in the second to last conserved portion of this loop of ZmPIP2;7 and ZmPIP2;8. Indeed ZmPIP2;8 confers robust water transport capacity in *Xenopus* oocyte swelling assay (**Fig. 4**). In conclusion we have, perhaps by serendipity, identified a new maize aquaporin ZmPIP2;8.

Materials and Methods

Aquaporin cloning and qRT-PCR. For root RNA isolation, 1 g of ground root tissue was added to 10 ml Trizol (Invitrogen) and

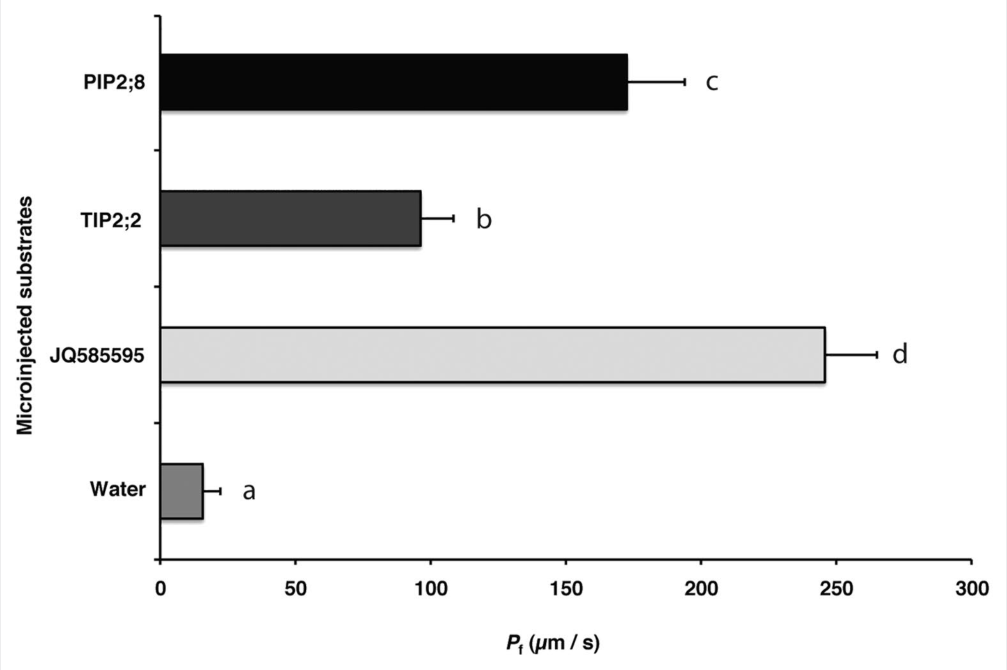

Figure 4. The water permeability of the oocytes microinjected with the cRNAs of ZmPIP2;8 and ZmTIP2;2, the cRNA of *Laccaria bicolor* aquaporin JQ585595 as the positive control and nuclease-free water as the negative control. Swelling assay was conducted in 0.2× hypotonic MBM after 48 h incubation in MBM solution at 18°C. Bar represents the mean of 10 replicates and error bar represents standard deviation. Letters indicate significant difference at $p < 0.05$.

incubated at room temperature for 5 min with frequent vortexing. Then 1 ml of chloroform was added vortexed for 15 sec, incubated at room temperature for 1 min and vortexed for 15 sec. The phases were separated by centrifugation at 20,190 × g for 10 min in a centrifuge. The top phase was collected and precipitated with 1 volume of isopropanol overnight at −80°C. The precipitate was pelleted by centrifugation at 11,950 × g for 10 min. The pellet was air-dried and resuspended in 200 μl sterile water. The samples were then further extracted using RNeasy mini spin columns with on-column DNase treatment as described above. RNA concentration and relative purity was assessed by absorbance at 260 and 280 nm with a spectrophotometer and integrity examined on a 1% (w/v) agarose gel with TBE buffer.

Primers for cloning were selected from the following sequences available at MaizeGDB (www.maizegdb.org)[10] for NIP1;1-GRMZM2G041980_TO2, for TIP2;2 GRMZM2G056908_T01 and PIP2-8 GRMZM2G432926_T01. The following primers were used to clone the aquaporins. TIP2-2-f CTC AGA GCC TCA GAG CGC CAG CCA AGT C, TIP2-2r-GGG AAA TGA ATA CAA GGA CGG AAT GGA ACA GAC C, PIP2 f-GCA ACA GGC AGG CCG CAC GCT CTT, PIP2 r-GAG ACA CGA AAT GCA TGC ACG GCG AAA AT, NIP1-1f-GGA GGA GGG CAG GAA GGA GGA GTT CGC, NIP1-1r-CGG CCC CAC CAC GTA CAC CCA GAT G. The Titanium One-step RT-PCR kit (Clontech) was used to produce cDNA, which was ligated to pGEM-T according to the manufacturer's protocol (Promega). cDNAs were sequenced and used to design custom Taqman primers and probes (see Appendix S4; Applied Biosystems). For measuring transcript levels during infestation, cDNA was synthesized from maize root RNA and qRT-PCR was performed as described in Lawrence et al.[7]

Protein sequence analysis. The SCR infestation-repressed *ZmPIP2;8* described in Lawrence et al.[7] is annotated in the maize genome and the transcript can be found at MaizeGDB (www.maizegdb.org)[10] as GRMZM2G432926_T01, which resides on chromosome 5 of the B73 RefGen version 2 genome between 38,210,596–38,202,808. A scan of the available maize aquaporin literature did not recover this sequence. The novel maize PIP2 contains an open reading frame, encoding a putative 283 amino acid protein. Since this is a newly described ZmPIP2, it was compared with other ZmPIP2 protein sequences, showing most similarity to ZmPIP2;2 at 86%. To determine whether it contained the conserved sequences novel to PIPs, two trans-membrane protein prediction programs were used, TMHMM Version 2.0 at www.cbs.dtu.dk/services/TMHMM/[25] and HMMTOP at www.enzim.hu/hmmtop.[26] The NIP1 proteins were aligned using CLUSTALW2 (www.ebi.ac.uk/Tools/msa/clustalw2). The position of the conserved amino acid motifs, Ar/R, P1-P5 and SDPs were determined by comparison to alignments of known H_2O_2 transporters from Hove and Bhave,[18] which include AtNIP1;2. These motifs were ascertained for ZmTIP2;2 and ZmPIP2;8 by aligning to ZmNIP1;1 and other ZmPIP2s.

Phylogenetic analysis of ZmPIP2;8. Alignment of sequences to ZmPIP2 proteins was performed using Megalign from lasergene 8.0 (DNASTAR). The phylogenetic trees were inferred using the Maximum Likelihood method based on the JTT matrix-based model.[27] Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join

Table 2. Comparison of conserved amino acids involved in pore formation of the ZmPIP2;8, ZmTIP2;2 and the NIP1s from rice, maize, *Arabidopsis* and soybean to the prototypical water channeling protein AQP1 and the prototypical glycerol channeling protein GlpF

	Ar/R filter				P1-P5 residues					SDPs H202							
Protein ID	**TM2**	**TM5**	**LE1**	**LE2**	**P1**	**P2**	**P3**	**P4**	**P5**	**TM3**	**TM3**	**TM3**	**TM3**	**TM5**	**HE**	**TM6**	**TM6**
ZmNIP1;1	W	V	A	R	F	S	A	Y	V	A	A	L	M	V	I	V	P
OsNIP1;1	W	V	A	R	F	S	A	Y	I	A	A	L	M	V	I	V	P
OsNIP1;2	W	V	A	R	F	S	A	Y	V	S	A	L	**V**	V	V	A	P
OsNIP1;3	W	V	A	R	F	T	A	Y	I	S	A	L	M	V	I	A	P
OsNIP1;4	W	V	A	R	F	S	A	Y	I	A	A	L	**L**	V	L	L	P
AtNIP1;1	W	V	A	R	F	S	A	Y	L	S	A	L	**L**	I	I	V	P
AtNIP1;2	W	V	A	R	F	S	A	Y	I	S	A	L	**L**	V	L	V	P
GmNOD26	W	V	A	R	F	S	A	Y	L	S	A	L	**L**	I	I	L	P
AtNIP2;1	W	V	A	R	F	S	A	Y	L	S	A	L	**L**	I	I	L	P
AtNIP3;1	W	I	A	R	F	S	A	Y	I	S	A	L	**V**	I	L	V	P
ZmPIP2;8	F	H	T	R	H	S	A	F	W	A	G	V	**F**	I	H	V	P
ZmTIP2;2	H	I	G	R	T	S	A	Y	W	A	A	L	V	I	N	V	P
AQP1	F	H	C	R	T	S	A	F	W								
GlpF	W	G	F	R	Y	D	K	P	L								

Conserved amino acids with AQP1 protein from humans (blue) and GlpF a glycerol channel protein from *E. coli* (red) are shown. The invariant R residue of LE2 is shown in black. Consensus signatures for H_2O_2 transporting aquaporins are as follows: Ar/R filter (H/F/W), (I/H/V), (A/T/G), (V/R); P1-P5 residues (T/Q/F), (A/S), A, (Y/F), (W/I); SDPs (S/A), (A/G), (L/V), (A/V/F/L), (I/V), (H/I/Q/L), (A/V), P.[18] The 8-SDP consensus residues from the H_2O_2 transporters conserved in the NIP1s, ZmTIP2;2 and ZmPIP2;8 are shown (green). Residues not found in the signatures of H_2O_2 transporters are underlined. AtNIP1;2 has been shown to be a functional H_2O_2 transporter.[20]

and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved nine amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 270 positions in the final data set. Evolutionary analyses were conducted in MEGA5.[28]

Subcloning *ZmTIP2;2* and *ZmPIP2;8* and testing in *Xenopus* oocyte swelling assay. The full-length cDNA of gene *ZmPIP* 2;8 of *Zea mays* was sub-cloned from pGEM vector into the restriction site of *Spe*I of the expression vector pXT7 containing the *T7* promoter and the 5' and 3' UTR of *Xenopus laevis* β-globin gene (provided by Dr. Warren Gallin, University of Alberta). The full-length cDNA of gene *ZmTIP2;2* of maize was sub-cloned into pXT7 at the restriction site of *Spe*I *and EcoRI*. The expression vector containing the target gene in the *T7* orientation was determined by sequencing and was linearized at the site of *Nde*I located downstream of 3' UTR of *Xenopus* β-globin gene. The linearized vector was used as the template for in vitro synthesis of capped RNA by using T7 RNA polymerase (mMESSAGE mMACHINE T7 kit, Ambion). The cRNA of aquaporin JQ585595 in *Laccaria bicolor* strain UAMH8232 was used as the positive control and nuclease-free water as the negative control. In *Xenopus* oocyte swelling assay, 10 ng of cRNA or nuclease-free water was microinjected into each healthy oocyte at Stage V-VI after being treated with 2 mg/ml collagenase in modified Barth's media for 2 h ([MBM] 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes-NaOH, 0.33 mM $Ca[NO_3]_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, Gentamicin sulfate 0.05 g/l, Penicillin G 0.1 g/l Na pyruvate 2.5 mM, pH 7.5) followed by a hypertonic solution of 100 mM potassium phosphate, pH 6.5 as described previously,[29,30] using an automatic nanoliter injector (Nanoject II, Drummond Scientific). After incubation in 200 mosmol MBM in scintillation vials at 18°C for 48 h, the injected oocyte was transferred into MBM in one well of a four-well Petri dish to take the initial image by an Olympus microscopy camera connected to an Olympus compound light microscope under 4× objective lens using the image software QCapture Pro 6.0 (QImaging) and then was transferred into D = 0.2 hypotonic MBM (40 mosmol) in the adjacent well and immediately the serial images were captured every 10 sec interval for 3 min, in order to track the changes in oocyte volume due to water influx. The diameter and surface area of oocytes were analyzed using ImageJ (V.1.44o, http://imagej.nih.gov/ij/index.html). The initial transmembrane volume flux and osmotic water permeability co efficiency (P_f) were calculated based on Zhang and Verkman[31] to represent the water permeability of the oocytes injected with cRNAs of each aquaporin gene. Statistic analysis was conducted using Origin 8.0 (OriginLab).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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